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# Sequential Production of Cytokines by Dengue Virus-Infected Human Peripheral Blood Leukocyte Cultures

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The study was undertaken to elucidate the sequence of appearance of T helper (Th)1- and Th2-type cytokines in human peripheral blood leucocyte cultures infected in vitro with dengue type 2 virus. Commercial sandwich enzyme-linked immunosorbent assay kits were used to assay the levels of tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-2, IL-4, IL-5, IL-6, and IL-10 in culture supernatants. Culture supernatants were also screened for the cytotoxic factor and the dengue virus titres determined. The cytokines that appeared in the culture supernatants on the first day post-infection (p.i.) were cytotoxic factor, TNF-α, IL-2, and IL-6; their levels were highest on the second day p.i. IFN-γ appeared on the second day with a peak on the third day p.i. The levels of these cytokines declined quickly, except for human cytotoxic factor (hCF) and IL-2. The cytokines that appeared later were IL-10 and IL-5 on the fourth day and IL-4 on the sixth day p.i. Dengue virus replicated in the peripheral blood leucocyte (PBL) cultures and was present throughout the course of the study. The findings of the present study show that dengue virus induced a predominant Th1-type cytokine response during the first 3 days of infection of PBL cultures that was replaced by a Th2-type response later. ***J. Med. Virol. 59:335–340, 1999.***


**KEY WORDS:** cytokines; dengue virus; pathogenesis; cytotoxic factor; Th1; Th2; TNF-α; IFN-γ; IL-2; IL-4; IL-5; IL-6; IL-10

## INTRODUCTION

Dengue virus produces both a mild self-limiting acute febrile illness (known as dengue fever) and also a life-threatening severe illness, known as dengue haemorrhagic fever. Dengue haemorrhagic fever has been classified into four grades on the basis of the clinical presentation and laboratory findings. The mildest is grade I and the most severe is grade IV [Nimmannitya, 1993]. The classical features of dengue haemorrhagic fever are increased capillary permeability, cerebral oedema, altered number and functions of leucocytes, increased haematocrit, and thrombocytopenia [Bhamarapravati, 1993]. Patients with dengue haemorrhagic fever grades III and IV may go into profound shock due to extensive plasma leakage into various serous cavities of the body. Despite extensive studies, the pathogenesis of dengue haemorrhagic fever is still not fully understood, though various suggestions have been made to explain the plasma leakage [Halstead, 1993; Kurane and Ennis, 1994].

$CD4^+$ helper T (Th) cells have two major subsets, Th1 and Th2 cells. Th1 cells secrete interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-beta (TNF-β) and are responsible for cell-mediated inflammatory reactions, delayed-type hypersensitivity, and tissue injury. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and are associated with help for antibody production by B cells. In a number of parasitic, fungal, bacterial, and viral infections, a Th1 response is linked to recovery from infection, whereas a Th2-type response tends to lead to severe pathology and exacerbation of the disease [reviewed by Mosmann and Sad, 1996]. Cross-regulation of the two clones is mediated by IL-10 and IFN-γ. Furthermore, TNF-α and IL-10 form an autoregulatory loop, in which TNF-α is an in-

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ducer of IL-10, and IL-10 is a down-regulator of TNF-α [Powrie and Coffman, 1993; van der Poll et al., 1994; Perez et al., 1995]. A few studies have reported the levels of IFN-γ, TNF-α, IL-1β, IL-2, and IL-6 in cases of dengue haemorrhagic fever [Kurane et al., 1991; Vitarana et al 1991; Hober et al., 1993]. In a recent study, a shift was observed from the predominant Th1-type response in patients with dengue fever to the Th2-type in severe cases with dengue haemorrhagic fever grade IV [Chaturvedi et al., 1999b]. Increased serum levels of IL-4 and IL-10 were observed mainly in cases with dengue haemorrhagic fever grades III and IV. The number of IL-2 and IL-6 positive sera was similar in cases with dengue fever and dengue haemorrhagic fever grade IV, but the mean value of IL-6 was significantly higher in the latter group. In contrast, the levels of IFN-γ and IL-2 were highest in cases with dengue fever and low in dengue haemorrhagic fever grade IV. TNF-α levels did not show a definite association. The cytokine levels to increase first were cytotoxic factor, IL-2, IFN-γ, and TNF-α whereas IL-4, IL-6, and IL-10 tended to emerge during the fourth to eighth day of the illness. A marked association of increased serum levels of IL-8 and transforming growth factor-beta (TGF-β) and the severity of dengue haemorrhagic fever were also observed [Raghupathy et al., 1998; Chaturvedi et al., 1999a; 1999b; Agarwal et al., personal communication]. Dengue virus also induces the production of cytotoxic factors [Shaio et al., 1995; reviewed by Chaturvedi et al., 1997]. A unique cytokine, cytotoxic factor, is produced by $CD4^+$ T cells in mice and humans. Cytotoxic factor appears to be pathogenesis-related proteins, capable of reproducing dengue haemorrhagic fever-like pathological lesions in mice, such as increased capillary permeability, cerebral oedema, and blood leukocyte changes [Chaturvedi et al., 1997; Mukerjee et al., 1997; Agarwal et al., 1998a; 1998b].

Dengue virus is known to stimulate human peripheral blood mononuclear cell cultures to induce production of various cytokines; the production of cytokines by cells from dengue immune donors is higher than that by nonimmune donors [Mukerjee et al., 1995; Yang et al., 1995; Chaturvedi et al., 1997; Mori et al., 1997; Hober et al., 1998]. The present study was undertaken to investigate the sequence of appearance of Th1- and Th2-type cytokines by infecting, with dengue virus, human peripheral blood leucocyte cultures obtained from dengue immune donors.

## MATERIALS AND METHODS

### Culture of Peripheral Blood Leukocytes

Blood was collected from two donors, both having IgG antibodies against dengue type 2 virus in their serum [Gentry et al., 1982]. From each of the donors, one unit (about 450 ml) peripheral venous blood was collected in a blood collection bag containing 63 ml anticoagulant (Baxter Healthcare Ltd., Thetford, Norfolk, England) and was centrifuged immediately at 1,800 × *g* for 15 min. The supernatant plasma was removed and the bag was filled with 10% Dextran 40 (Dextran sulfate MW 40,000 Dalton) solution in 0.9% sodium chloride (Pharmaceutical Solution Industries Ltd., Jeddah, Saudi Arabia), after which the cells were evenly resuspended and centrifuged again at 1,800 × *g* for 15 min to sediment the red blood cells at the bottom of the bag. The peripheral blood leucocyte-rich fluid was collected and the cells were washed thrice with Hanks basal salt solution. The cells were resuspended in minimum essential medium (MEM) containing 5% fetal calf serum. Total and differential cell counts were carried out and on this basis the cells were calculated to contain $1 \times 10^6$ mononuclear cell/ml. The cells were distributed in Nunclon Delta culture tubes (Nunc, Denmark) and were cultured at 37°C in presence of 5% $CO_2$ in air.

### Dengue Virus

Dengue type 2 virus, strain P23085 (in the form of infected adult mouse brain suspension) was used [Chaturvedi et al., 1978]. The dose of the virus was 1,000 $LD_{50}$ per $1 \times 10^6$ mononuclear cells (lymphocytes + monocytes) and on the basis of the differential leucocyte counts this represented about 45,000 monocytes. The same dose of the virus was used in similar experiments reported earlier [Mukerjee et al., 1995]. The controls were inoculated with a similar dilution of normal mouse brain suspension. The culture supernatants collected at different times post-infection (p.i.) were assayed for the virus titre by intracerebral inoculation of groups of mice in serial 10-fold dilutions of the culture supernatants. The virus titre was calculated by the method of Reed and Muench [1938] and expressed as $\log_{10}$ $LD_{50}$ per millilitre of the culture supernatants.

### Assay of Cytokines

The cytokine levels in the culture supernatants were assayed by sandwich- enzyme-linked immunosorbent assay (ELISA) using commercial kits (Immunotech, Coulter Co. France). Assays were carried out on undiluted culture supernatants according to the instructions of the manufacturer. All the tests were set up in duplicate and the data was analyzed by Genesis Windows Software for microplate-based assays (Labsystems). The minimum detectable concentrations in this assay were 5 pg/ml TNF-α, 8 pg/ml IFN-γ, 5 pg/ml IL-2, 5 pg/ml IL-4, 1 pg/ml IL-5, 3 pg/ml IL-6, and 5 pg/ml IL-10.

### Assay of Cytotoxic Factor

Culture supernatants were screened for the presence of cytotoxic factor by the cytotoxicity assay as described elsewhere [Agarwal et al., 1998b]. Briefly, equal volumes of culture supernatants and a single cell suspension of normal mouse spleen ($2 \times 10^6$ cells) were mixed in 96-well Perspex plates and kept at 4°C for 1 hr. Viability of the cells was screened using Trypan blue dye and the percentage of nonviable cells was expressed after deduction of background values obtained from untreated control cells.

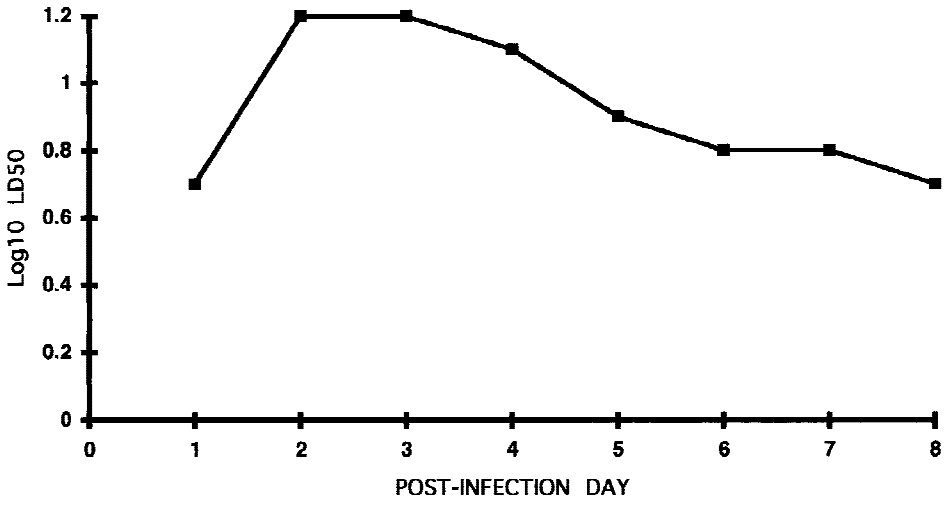


Fig. 1. Titre of dengue virus type 2 in the culture supernatants of peripheral blood leucocyte cultures at different periods following virus inoculation. Each point represents an average for four cultures.

### Plan of Study

The peripheral blood leucocyte culture tubes were divided into three groups. One group of tubes were inoculated with 1,000 $LD_{50}$ of the dengue virus/1 × $10^6$ mononuclear cells, the second group of the tubes were inoculated with similar dilutions of normal mouse brain suspension for controls and uninoculated culture tubes were included for the background values. All the culture tubes were incubated at 37°C in presence of 5% $CO_2$. At 0 hours and daily from day 1 to 8 after inoculation, three culture tubes from each of the groups were harvested and the culture supernatants collected were distributed in small aliquots and stored at −70°C until they were tested. After deduction of the background values the mean values ± SD obtained from thrice repeated experiments are presented below.

## RESULTS

### Dengue Virus Titres in Peripheral Blood Leucocyte Cultures

The culture supernatants from three peripheral blood leucocyte cultures taken on different days were titrated for dengue virus. The average titres of the virus presented in Figure 1 show that the virus replicated in the peripheral blood leucocytes and was present throughout the period of study with a peak titre on the second day of infection.

### Early Cytokines

***Tumor necrosis factor-α.*** The data summarised in Figure 2 show the mean values of the concentration of TNF-α in the culture supernatants of the peripheral blood leucocyte cultures on different days of infection with dengue virus. TNF-α appeared on the first day p.i. (36 ± 12 pg/ml) and the maximum mean value of 236 ± 55 pg/ml was observed on the second day. This measurement was followed by a sharp decline, with the mean value being 18 ± 7 on the 5th day p.i. None of the control culture supernatants had detectable amounts of TNF-α.

***Interferon-γ.*** As can be seen in Figure 2, IFN-γ was detectable in the culture supernatants on the second day p.i. (132 ± 24 pg/ml), reaching a peak mean value of 528 ± 102 pg/ml on the third day. After a sharp decline, IFN-γ was not detectable from day 6 onwards. IFN-γ was not detectable in the control culture supernatants.

***Interleukin-2.*** The mean value of IL-2 in the control culture supernatants was 15–20 pg/ml on different days of the culture (Fig. 2). In the dengue virus-infected cultures, the mean value of IL-2 on the first day p.i. was 329 ± 75 pg/ml, with a peak value of 618 ± 125 pg/ml on the second day. This measurement was followed by a gradual decline and on the eighth day p.i., the mean value was 163 ± 46 pg/ml (Fig. 2).

***Interleukin-6.*** The mean culture supernatants levels of IL-6 in the controls was 20–50 pg/ml on the different days of the culture. The mean concentration of IL-6 was 584 ± 95 pg/ml in the culture supernatants collected on the first day p.i., with a peak value of 1,725 ± 217 pg/ml on the second day p.i. Figure 2 shows a marked decline in IL-6 levels from the fourth day and was 88 ± 24 pg/ml on the eighth day.

***Cytotoxic factor.*** The culture supernatants from peripheral blood leucocyte cultures were screened for the presence of cytotoxic factor by cytotoxicity assay. A day-wise distribution presented in Figure 3 shows the presence of cytotoxic factor from the first to the eighth day of infection with dengue virus. Further, higher cytotoxic activity was observed during the second to fourth day of the infection. The culture supernatants of the control cells collected during the first to eighth day of culture had negligible cytotoxic activity ($P \leq .001$).

### Late Cytokines

***Interleukin-4.*** The findings presented in Figure 4 show that IL-4 appeared in the culture supernatants of

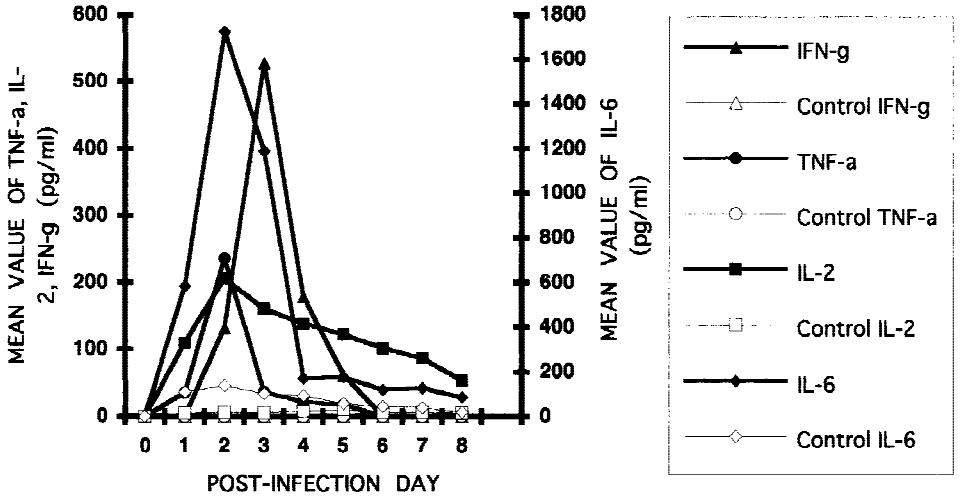


Fig. 2. Levels of cytokines in the culture supernatants of dengue virus-infected human peripheral blood leucocyte during the early phase of infection. Culture supernatants collected were examined for the cytokine concentrations by sandwich enzyme-linked immunosorbent assay using commercial kits. The mean values of the data have been presented.

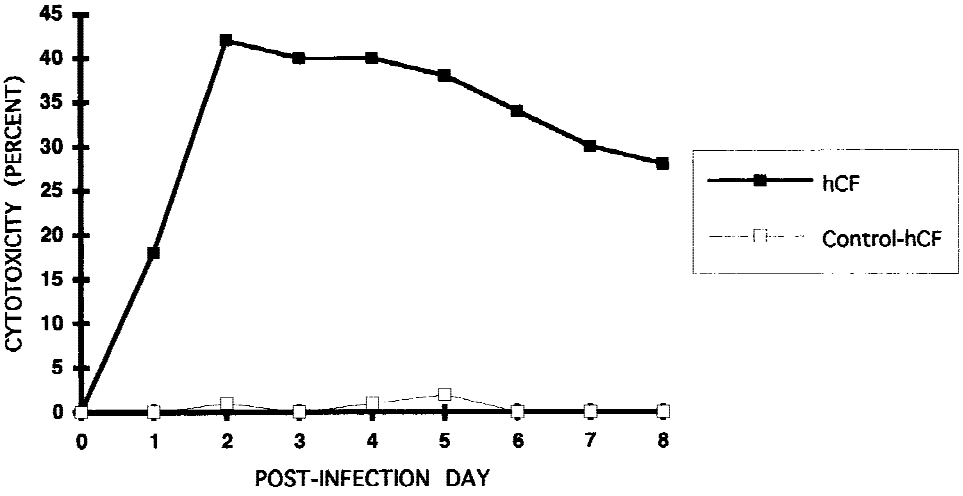


Fig. 3. Presence of cytotoxic factor in the culture supernatants of dengue virus-infected human peripheral blood leucocyte during the early phase of infection. Culture supernatants collected were screened for cytotoxic factor by cytotoxicity assay using normal mouse spleen cells as target. Percentages of the target cells killed were recorded after deducting the background values. The mean values of the data have been presented.

peripheral blood leucocyte on the sixth day (103 ± 19 pg/ml), with the maximum value of 216 ± 34 pg/ml on the seventh day p.i. IL-4 was absent in all the control peripheral blood leucocyte cultures.

***Interleukin-5.*** IL-5 appeared in the culture supernatants of peripheral blood leucocyte cultures on the fourth day (251 ± 57 pg/ml) after dengue virus infection, reaching peak levels (558 ± 96 pg/ml) on the 5th day. IL-5 persisted until the end and was 425 ± 88 pg/ml on the eighth day (Fig. 4). The control culture supernatants contained negligible amounts of IL-5.

***Interleukin-10.*** IL-10 was absent in all the control culture supernatants. The data summarised in Figure 4 show that IL-10 was present in the culture supernatants on the fourth day p.i. (98 ± 24 pg/ml) and the peak mean value of 514 ± 84 pg/ml was found on the 7th day after dengue virus infection.

## DISCUSSION

The findings of the present study show a shift from the predominant Th1-type response observed during the early phase (within the first 3 days) of the infection of the peripheral blood leucocyte cultures by dengue virus, to the Th2-type in the later phases (day 4 onwards). The level of IL-6 increased sharply, reaching a peak (1725 ± 217 pg/ml) on the second day and then declined quickly but persisted along with IL-2, cytotoxic factor, and dengue virus throughout the course of the study. It was observed that cytotoxic factor, IL-2, and IL-6 appeared with fast kinetics, IFN-γ with inter-

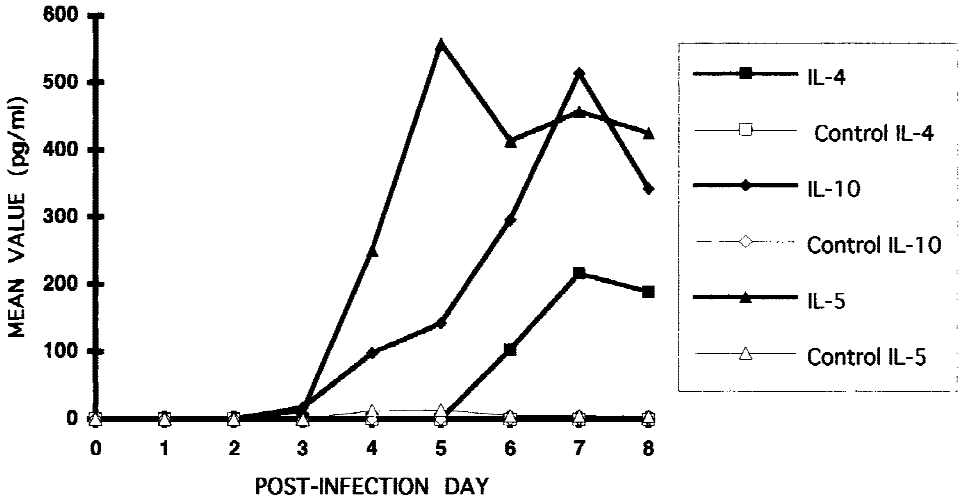


Fig. 4. Levels of cytokines in the culture supernatants of dengue virus-infected peripheral blood leucocyte during the later phase of infection. Culture supernatants collected were examined for the cytokine concentrations by sandwich enzyme-linked immunosorbent assay using commercial kits. The mean values of the data have been presented.

mediate kinetics, and IL-5, IL-10, and IL-4 with slow kinetics, IL-4 being the slowest. A similar response had been observed in Th cells stimulated by staphylococcal enterotoxin B [Assenmacher et al., 1998].

These results suggest an initial bias toward Th1-type reactivity (elevated levels of IL-2, IFN-γ, and TNF-α), followed by a shift in favour of Th2-type reactivity (high levels of IL-4, IL-5, and IL-10). Interestingly, IL-6 showed up as an early cytokine in this study; although IL-6 is considered a Th2-type cytokine because of its role in inducing the differentiation of B cells to plasma cells and the stimulation of antibody production [Romagnani, 1994], it also plays active roles in inflammatory responses. IL-6 stimulates the production of acute phase proteins and is responsible for many of the local and systemic changes observed in acute inflammatory reactions [Akira et al., 1993]. In this study, although IL-6 declined on day 4, it continued to persist, albeit at lower levels.

In a few studies, human peripheral blood leucocyte cultures have been used to study cytokine production during dengue virus infection [Mukerjee et al., 1995; Chaturvedi et al., 1997; Mori et al., 1997; Hober et al., 1998]. Recently, Mori et al. [1997] have used a double immunocytochemical technique to demonstrate cytokine production by dengue virus-stimulated human T lymphocytes. They have shown an increase in the number of IFN-γ, IL-2, IL-4, and TNF-β-positive cells 2–3 days after stimulation with dengue virus antigen, which is similar to the findings of the present study. However, the number of such cytokine-positive cells may not indicate the amount of cytokine produced, as this may depend on factors that trigger or down-regulate the cytokine-producing cells.

Cytotoxic factor is present in about 90% of the patients with dengue fever/dengue haemorrhagic fever, with peak amounts during the first 4 days of illness and in the most severe cases with dengue haemorrhagic fever grade IV [Chaturvedi et al., 1999a]. Further, ex vivo culture of peripheral blood mononuclear cells ($CD4^+$ T cells) from such patients produce cytotoxic factor but the nature of cytotoxic factor-producing cells is not known [Agarwal et al., 1998a, 1998b]. The pathogenic role of cytotoxic factor has been established by producing lesions in mice similar to those seen in dengue haemorrhagic fever, for example, increased capillary permeability and cerebral oedema [reviewed by Chaturvedi et al., 1997].

Cytokines act within a cross-regulating network, for example, Th1 and Th2 cells are cross-regulated by IL-10 and IFN-γ. Furthermore, TNF-α and IL-10 form an autoregulatory loop, in which TNF-α is an inducer of IL-10, and IL-10 is a down-regulator of TNF-α. The free radicals, nitrite and peroxynitrite, directly up-regulate production of IL-1β, TNF-α, IL-8, and hydrogen peroxide in macrophages, whereas TNF-α induces the production of IL-6, IL-8, and IL-10 [reviewed by Cerami, 1992; Merrill and Benveniste, 1996].

The production of cytokines could be due either to sequential generation of different clones of Th cells secreting different cytokines or the same cell secreting different cytokines at different stages of activation. A functional analysis of separated staphylococcal enterotoxin B-activated live cells showed that a single cell produces the Th1-cytokines and the Th2-cytokines sequentially [Assenmacher et al., 1998]. With the available data, it may be proposed that dengue virus induces the production of cytotoxic factor, which generates free radicals [reviewed by Chaturvedi et al., 1997], which in turn induce generation of a Th1-type cytokine response followed by that of a Th2-type response. This proposal supports the finding of a shift of Th1-type response to Th2-type in patients with dengue haemorrhagic fever [Chaturvedi et al., 1999b]. Whether this response involves a single Th cell or different clones of Th cells is not known.

A similar Th1 to Th2 switch has been described in HIV infection; HIV-positive patients progressing to AIDS have increased IL-4 responses and decreased IFN-γ responses [Clerici and Shearer, 1993], both of which are hallmarks for increased Th2-type reactivity and decreased Th2-type reactivity respectively. The primary protective acquired immune defense against intracellular infections is the Th1-mediated response: a decrease in Th1-type reactivity generally results in exacerbation of the disease and immunopathological consequences. We suggest that a Th1 to Th2 shift in dengue-infected patients is associated with increased severity of the disease, followed by death. Much work remains to be done to elucidate the pathogenesis of dengue.

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